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# Characterization of an Immunodominant Antigenic Epitope from *Trypanosoma cruzi* as a Biomarker of Chronic Chagas' Disease Pathology

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**Nowadays, the techniques available for chronic Chagas' disease diagnosis are very sensitive; however, they do not allow discrimination of the patient's clinical stages of the disease. The present paper describes that three out of the five different repeats contained in the *Trypanosoma cruzi* TcCA-2 membrane protein (3972-FGQAAAGDKPPP, 6303-FGQAAAGDKPAP, and 3973-FGQAAAGDKPSL) are recognized with high sensitivity (>90%) by sera from chronic Chagas' disease patients and that they are not recognized by sera from patients in the acute phase of the disease. A total of 133 serum samples from chagasic patients and 50 serum samples from healthy donors were tested. In addition, sera from 15 patients with different autoimmune diseases, 43 serum samples from patients suffering an infectious disease other than Chagas' disease, and 38 serum samples from patients with nonchagasic cardiac disorders were also included in this study. The residue 3973 peptide shows a specificity of >98%, as it is not recognized by individuals with autoimmune and inflammatory processes or by patients with a nonchagasic cardiomyopathy. Remarkably, the levels of antibody against the 3973 epitope detected by the sera from Chagas' disease patients in the symptomatic chronic phase, involving cardiac or digestive alterations, are higher than those detected by the sera from Chagas' disease patients in the indeterminate phase of the disease. It is suggested that the diagnostic technique described could also be used to indicate the degree of pathology. The amino acids F, Q, and DKP located in the peptide at positions 1, 3, and 8 to 10, respectively, are essential to conform to the immunodominant antigenic epitope.**

Chagas' disease (ChD) is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted by hematophagous Reduviidae vectors and infects many wild animal reservoirs. Despite the intensive programs implemented to control the illness-transmitting vectors, this neglected tropical sickness affects about 8 million people located in the poorest regions of developing countries in Latin America and the Caribbean (31, 32). In these areas, there is a high level of exposure and transmission due to the poor housing and living conditions, together with ecological factors that have led to the adaptation of the triatomine vectors. In addition to congenital transmission, blood transfusion, and organ transplantations, there are other ways of infection, such as oral transmission through ingestion of contaminated food, currently considered an important route of transmission (2). Due to the increase of immigrants, high numbers of imported ChD cases are now being detected in areas where the disease is not endemic (18, 24). Therefore, ChD is acquiring relevance from a public health point of view both in countries where the disease is endemic and in countries where it is not endemic, where children and pregnant women are the most vulnerable populations (23).

The disease courses with different clinical forms. The acute phase appears shortly after infection. In this phase, the parasite can be visualized in the bloodstream. In the absence of treatment, the acute phase is followed by an indeterminate stage in which the parasites persist in specific tissues (28). In about one-third of these individuals, the infection leads to a symptomatic chronic phase, characterized by cardiac and digestive involvement (8, 28). Cardiac manifestations of the disease include arrhythmias, electrocardiographic abnormalities, cardiomegaly, and/or systolic dysfunction (29, 30). Digestive manifestations of the disease include aperistalsis megaesophagus and megacolon. Although these clinical manifestations are usually not highly severe, they are associated with morbidity (27).

Nowadays, ChD is diagnosed mostly by serological tests. These tests are based on the detection of specific antibodies against homogenates of the parasite's total proteins or combinations of recombinant proteins as antigens. Although these techniques are very sensitive, they do not allow differentiation of the clinical phases of the disease. Moreover, these techniques do not seem to be reliable tools for monitoring the evolution of the disease in patients after treatment (3, 5, 26, 37). In this context, potential biomarkers for therapeutic efficacy have recently been described (9, 38). Detection of the parasite in the bloodstream of treated patients by PCR does not allow prediction of treatment success, since even repeated negative PCR results do not necessarily indicate parasitological cure (31). However, diagnosis by PCR may constitute a tool to highlight the impact of treatment and the reduction of the parasitic load (25).

**Received** 4 November 2011 **Returned for modification** 17 November 2011
**Accepted** 28 November 2011

**Published ahead of print** 7 December 2011

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doi:10.1128/CVI.05566-11

**TABLE 1** Characteristics of the population under study that came from areas of endemicity and that are Spanish residents

| Subject group[a] | Mean (range) age (yr) | No. (%) of males/females | No. (%) of patients, origin |
|---|---|---|---|
| HD ($n = 30$) | 30.6 (24–50) | 6 (20.7)/23 (79.3) | 26 (86.6), Bolivia; 4 (13.3), Ecuador |
| IND ($n = 28$) | 31.5 (12–47) | 8 (28.6)/20 (71.4) | 27 (96.4), Bolivia; 1 (3.6), Paraguay |
| CCC ($n = 38$) | 40.0 (28–74) | 16 (42.1)/22 (57.9) | 38 (100.0), Bolivia |
| DIG ($n = 21$) | 33.7 (12–54) | 8 (38.0)/13 (62.0) | 21 (100.0), Bolivia |

[a] HD, healthy donors; IND, chronic Chagas' disease patients at the indeterminate clinical phase of the disease; CCC, chronic cardiac Chagas' disease patients; DIG, chronic Chagas' disease patients with abnormalities in the gastrointestinal tract.

In the *T. cruzi* parasite, a significant number of proteins containing large tandem repeat domains have been shown to have significant immunological relevance, since most of them are antigenic molecules (12). The antigens bearing amino acid tandem repeats seem to possess a significant degree of antigenicity and to be targets of B-cell responses. It seems that the reactivity of chagasic patients' serum samples against these antigens has a great degree of specificity and sensitivity (10, 15, 36). It has been described that the immunodominant membrane protein of *T. cruzi* TcCA-2 bearing different repeated epitopes of 12-mer in length (4), as well as its homologs, the T-cell receptor 39 (TCR39) (15) and B13 antigens (13, 15), is recognized with high sensitivity by sera from ChD patients (1, 7). The TcCA-2 protein also contains the TcMe (*T. cruzi* specific epitope) motif, which has been described to be involved in the internalization of the parasite into the host cell (22a). The B13 protein contains T-cell epitopes located in a tandemly repetitive fashion and has low homology with multiple epitopes contained in the human cardiac myosin (1, 17). The involvement of cross-reactivity between cardiac myosin and B13 in the pathogenesis of chronic cardiac ChD has been suggested (6, 17).

The aim of this work was to analyze the reactivity of sera from chagasic patients against the different repeated epitopes present in the TcCA-2 protein. The level of recognition of the most immunodominant repetitive epitope, epitope 3973 (FGQAAAGDKPSL), from TcCA-2 by sera from adult ChD patients having different clinical forms of the disease is described. The existence of a differential reactivity against the 3973 peptide of sera from symptomatic and nonsymptomatic Chagas' disease patients is also demonstrated. Furthermore, we have identified the minimal residues that conform to the antigenic epitope.

## MATERIALS AND METHODS

**Human sera.** Following WHO criteria, ChD diagnosis was determined using two different commercial serological tests (enzyme-linked immunosorbent assay [ELISA; Bioelisa Chagas; Biokit, Barcelona, Spain] and indirect immunofluorescence assay [IFI; Inmunofluor Chagas; Biocientífica, Argentina]). According to diagnostic test results, a total of 133 serum samples from chagasic patients and 50 serum samples from healthy donors (HDs) were assayed. Thus, serum samples from 87 chronic ChD adult patients (Chronic Ch) and 30 control serum samples from healthy adult donors were collected at the Virgen de la Arrixaca Hospital (Murcia, Spain). These people came from areas of endemicity and were residents of Spain, where *T. cruzi* reinfection does not occur (Table 1). Patients were considered to be at the indeterminate phase (IND; $n = 28$) when they were seropositive with no evidence of cardiac disorder (following clinical criteria and radiological, electrocardiographic, and transthoracic echocardiography analyses) or gastrointestinal tract disorder. Patients with chronic Chagas' cardiomyopathy (CCC; $n = 38$) were catalogued into stages G1 to G3, following the Kuschnir classification, according to clinical criteria and radiological, electrocardiographic, and transthoracic echocardiography analyses (19). The digestive form (DIG; $n = 21$) was identified when megaesophagus and/or megacolon abnormalities in the gastrointestinal track were detected by esophagogram and barium enema analyses. Serum samples from 11 patients with orally acquired acute ChD (Acute Ch), 35 adults with chronic ChD diagnosed by ELISA (IgG and IgM) and indirect hemagglutination tests, and 20 healthy donors who live in the area of endemicity were collected at the Instituto de Medicina Tropical (Caracas, Venezuela).

Serum samples from patients with different autoimmune disorders or patients suffering an infectious disease other than the Chagas' disease were also included in this study as controls. Thus, sera from 15 patients from areas of nonendemicity suffering autoimmune diseases (systemic lupus erythematosus [SLE; $n = 6$], celiac disease [$n = 4$], and rheumatoid arthritis [Ai; $n = 5$]) were collected at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), and 43 serum samples from patients with an infectious pathology (leishmaniasis [Leish; $n = 21$], tuberculosis [Tuberc; $n = 11$], and malaria [$n = 11$]) were collected from the Centro para Estudios sobre Malaria-IAES-MPPS (Caracas, Venezuela). In addition, serum samples from patients with nonchagasic cardiac disorders (NChCard; $n = 38$), including dilated idiopathic cardiomyopathy ($n = 13$), ischemic cardiomyopathy ($n = 8$), valvular cardiomyopathy ($n = 7$), heart failure with normal left ventricular ejection fraction ($n = 6$), heart transplantation graft acute rejection ($n = 2$), evolving myocardial infarction ($n = 1$), and acute viral myocarditis ($n = 1$), were included in this study.

**Ethical considerations.** The protocols were approved by the ethical committees of the Instituto de Medicina Tropical (Caracas, Venezuela) and Hospital Virgen de la Arrixaca and Consejo Superior de Investigaciones Científicas (Spain). A signed informed consent was obtained from all individuals prior to their inclusion in the study.

**Synthetic peptides.** Peptides were synthesized by the simultaneous multiple-peptide solid-phase method. The peptides were assembled using the standard *tert*-butoxycarbonyl (tBoc) solid-phase peptide synthesis (SPPS) strategy on *p*-methylbenzhydrylamide (MBHA) resin. Purity was checked by high-performance liquid chromatography (HPLC), and the correct composition was verified by mass spectrometry (16). The peptides used in this study are listed below: 3972 (FGQAAAGDKPPP), 6303 (FGQAAAGDKPAP), 3973 (FGQAAAGDKPSL), 3963 (FGQAAAGDKLSL), 6173 (FGQAAAGGKPSL), 6175 (AGQAAAGDKPSL), 6380 (FAQAAAGDKPSL), 6383 (FGAAAAGDKPSL), 3964 (FGQAAAGHKPAP), 3965 (FGQAAEGDKPPP), 3966 (FGQAAAADKPSL), 3974 (FGQAAAGDLPSP), 6174 (FGQAAAGDGPSL), 6382 (AAQAAAGDKPSL), 6304 (FAQAAAADKPSL), 6172 (FGQAAAGERPSL), 6381 (AAAAAAGDKPAA), 6302 (FAQAAAADKPAA), 6385 (QAAAGDKPSLFG), and 6384 (AAGDKPSLFGQA).

**ELISA measurement.** The ELISA described here is a modification of a method described in detail by Thomas et al. (35). Briefly, ELISA 8-well strips (Nunc-Immuno module F16; Roskilde, Denmark) were coated with 100 $\mu$l of peptide (10 $\mu$g/ml in phosphate-buffered saline [PBS]) and dried stored at −20°C until use.

Subsequently, the wells were washed twice with 200 $\mu$l of PBS–0.05% Tween 20 (Sigma) and incubated for half an hour with blocking solution (2.5% nonfat dried milk powder in PBS). Afterward, patient sera at 1/400, 1/800, and 1/1,600 dilutions were added to the antigen-coated wells and

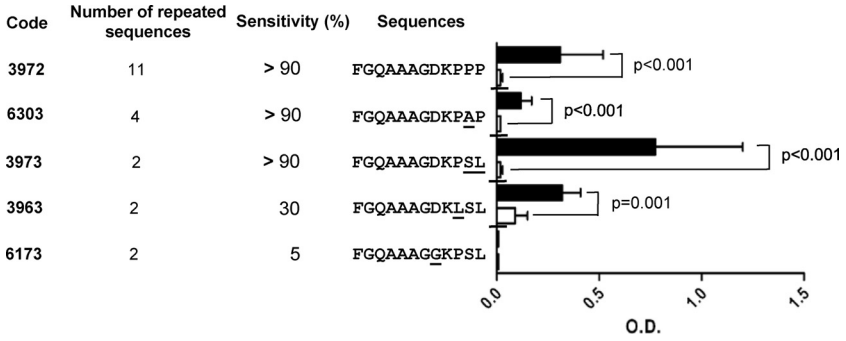


**FIG 1** Reactivity of sera from Chagas' disease patients against five 12-mer peptides present in the *T. cruzi* TcCA-2 protein. The amino acid divergences of the different peptides representing the most abundant repeat are underlined. Data are expressed in optical density (O.D.) values. The horizontal bars represent the median optical density values and standard deviations obtained against 35 sera from chagasic patients (■) and 20 serum samples from healthy donors (□). Statistically significant differences ($P < 0.05$) are indicated. Sera were assayed at a 1:400 dilution.

the reaction mixture was incubated for 1 h at 37°C. As a secondary antibody, affinity-isolated goat anti-human IgG antibody, peroxidase conjugated (Biosource, Spring Valley, NY), was used at a dilution of 1:2,000. The reaction mixture was incubated for 1 h at 37°C. After the reaction mixture was washed, the reaction was developed using 3,3′,5,5′-tetramethylbenzidine (TMB; 0.4 g/liter) and hydrogen peroxide (0.02%) (1:1) for 10 min at room temperature. The absorbance was measured at 620 nm. Serum samples were assayed in triplicate. Hyperimmune sera from mice immunized with each peptide coupled to keyhole limpet hemocyanin (KLH; Sigma) and sera from a healthy donor were included in all plates as positive- and negative-control sera, respectively.

**Statistical analysis.** The Mann-Whitney U test was used to carry out comparisons among groups of patients. Pearson correlation analyses were used to evaluate the correlation among levels of antibody against the 3973 peptide and 6302 peptide. The relation between level of antibody against the 3973 peptide and Kuschnir stage was tested with the Jonckheere-Terpstra statistic on trend. Statistical significance was assigned at a two-sided $P$ value of $\leq 0.05$. Statistical analyses were performed using the SPPS statistical package, version 15.0 (SPSS Inc., Chicago IL).

## RESULTS

**Antigenicity and sensitivity of natural peptides contained in TcCA-2 antigen.** The reactivity of sera from 35 Latin American residents at the chronic phase of ChD against a series of peptides that correspond to different 12-mer tandem repeats present in the TcCA-2 immunodominant antigen of *T. cruzi* (GenBank accession number EAN97076) was analyzed. These tandem repeats differ in their amino acid compositions, affecting 1 or 2 amino acids located at position 8, 10, 11, or 12 of the repeat. The number of repeats ranges from 2 to 11. Thus, five peptides of 12 amino acids in length that correspond to these repeats were synthesized. As shown in Fig. 1, peptides 3972, 6303, and 3973 are recognized by at least 90% of chagasic patient sera. Sequence divergences among these three natural peptides are on positions 11 and 12. Peptide 6303 differs from peptide 3972 in the amino acid located at position 11, as it bears alanine instead of proline. Peptide 3973 bears serine and leucine amino acids at positions 11 and 12 instead of the prolines in peptide 3972.

A single amino acid divergence in another position of the repeat resulted in an important reduction of the percentage of patients' sera that recognized the peptide. Thus, peptide 3963 was recognized by only 30% of the chronic chagasic sera. This drop in the percentage of patients' sera that recognized the peptide is due to the difference in amino acid composition at position 10, as peptide 3963 bears lysine instead of proline in peptides 3972, 6303, and 3973. A drastic decrease in the percent recognition was detected when peptide 6173 was tested. This peptide contains a single modification that affects position 8 of the repeat. Peptide 6173, which bears glycine instead of aspartic acid, was recognized by only 5% of the serum samples. These data indicate that small differences in the amino acid composition of the repeats strongly influence the antigenic nature of these sequences. The highest reactivity was detected when the repeat present in peptide 3973 was tested (Fig. 1). Consequently, the 3973 peptide was selected for further studies.

**Specificity against 3973 peptide and correlation of serum reactivity with sickness severity.** The reactivity of a larger number of serum samples from chagasic patients against the 3973 peptide was analyzed. It was also determined whether the reactivity against the 3973 peptide is correlated with the disease form and the cardiac severity phase and whether 3973-specific antibodies are present in the sera from patients with other *T. cruzi*-related infectious diseases and with other related pathologies. Sera from 87 chronic ChD adult patients and 30 control serum samples from healthy adult donors from areas of endemicity (at present, Spanish residents) were assayed for reactivity against peptide 3973. Sera from 11 patients at the orally acquired acute phase that were collected at the Instituto de Medicina Tropical (Caracas, Venezuela) were also assayed for reactivity against the same peptide. The results show that most chronic ChD patients (94.25%) had antibodies against the 3973 epitope and that in most of them the reactivity was high (Fig. 2A). However, the sera from the above-mentioned patients at the acute phase did not recognize the 3973 peptide (Fig. 2A), an indication that the presence of anti-3973 antibodies is directly correlated with the establishment of the chronic pathology. The high level of specificity (>98%) of recognition of peptide 3973 by the sera from chagasic patients at the chronic phase was clearly shown by the observation that none of the sera from patients with leishmaniasis ($n = 21$), tuberculosis ($n = 11$), and malaria ($n = 11$) recognized this peptide (Fig. 2A).

To address the question whether the anti-3973 reactivity could correlate with the different phases of the sickness in terms of the pathology severity, the presence of antibodies in patients presenting different clinical forms of the disease was evaluated. Thus, sera from patients at the indeterminate phase (IND; $n = 28$) and from patients at the chronic phase who presented chronic cardiac dam-

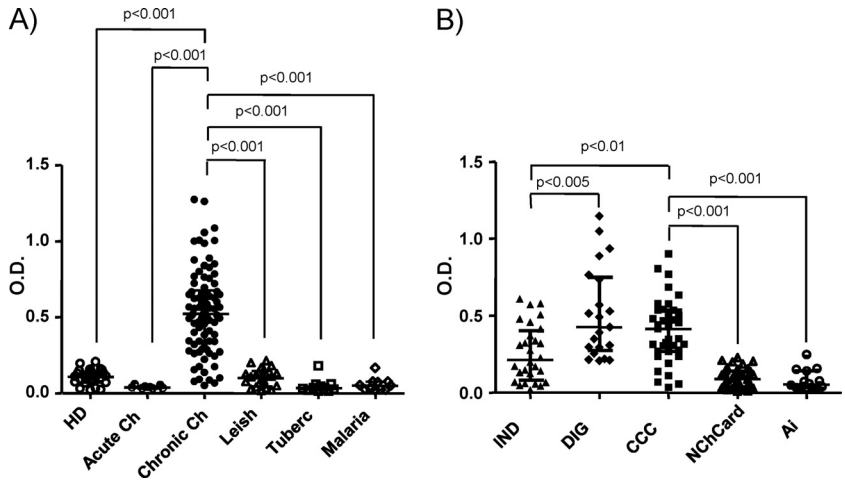


**FIG 2** Specificity of IgG antibodies against 3973 peptide. The IgG reactivity against the 3973 peptide was measured by ELISA. Data are expressed as the optical density (O.D.). Horizontal lines on each group represent the median and interquartile range. Statistically significant differences ($P < 0.05$) are indicated. (A) Sera from 30 healthy donors (HD, ○), 11 acute Chagas' disease patients (Acute Ch, ◓), 87 chronic Chagas' disease patients (Chronic Ch, ●), 21 leishmaniasis patients (Leish, △), 11 tuberculosis patients (Tuberc, □), and 11 malaria patients (◇). The sera were assayed at 1:800 dilution. (B) Sera from 28 patients with the indeterminate form (IND, ▲), 21 patients with the digestive form (DIG, ◆), 38 patients with the cardiac form (CCC, ■), 38 patients with nonchagasic cardiac disorders (NChCard, △), and 15 patients with autoimmune disorders (rheumatoid arthritis [Ai], ◓). Sera were assayed at 1:1,600 dilution.

age (CCC; $n = 38$) or digestive alterations (DIG; $n = 21$) were analyzed by ELISA. The data indicate that the reactivity against peptide 3973 detected in the sera from patients with ChD in the chronic form (both DIG and CCC) is higher, with statistical significance, than that detected in sera from patients with the IND form (Fig. 2B). No statistically significant differences were observed when the reactivity against 3973 in DIG patients was compared to that in CCC patients. There was no correlation between the patients' age and the level of reactivity against the 3973 peptide (data not shown).

In order to rule out the possibility that the recognition of the 3973 epitope is due to other clinical manifestations of cardiac damage or inflammatory processes not associated with ChD, sera from patients with cardiomyopathy or other heart disorders (NchCard) and sera from patients with autoimmune pathologies such as SLE, celiac disease, and rheumatoid arthritis (Ai; $n = 15$) were assayed for reactivity against peptide 3973. As shown in Fig. 2B, the reactivity against 3973 is significantly higher in the sera from chagasic patients having clinical cardiac or digestive manifestations than in the sera from patients with nonchagasic cardiac disorders and patients having autoimmune and inflammatory processes such as SLE, rheumatoid arthritis, or celiac disease. In addition, we found a positive trend ($P < 0.01$, Jonckheere-Terpstra test) in the relation between the reactivity against peptide 3973 by chronic ChD patient sera and the severity of the cardiac damage determined by the Kuschnir classification value (G0 to G2/G3) or the presence of digestive disorders ($P = 0.001$) (Fig. 3). Thus, the data presented suggest that the detection of antibodies against the *T. cruzi* 3973 peptide in sera from chronic chagasic patients may well be used as a serological marker for follow-up of the evolution of the disease from an asymptomatic stage to a cardiac or digestive symptomatic form, thus having a prognosis value.

**Identification of amino acid composition and localization of the epitope contained in the 3973 peptide that is recognized by sera from chagasic patients.** To identify the position and nature of the amino acids present in the 3973 epitope to which the antibodies are directed, a series of peptides bearing amino acid substitutions along the sequence was synthesized and assayed by ELISA. Simple, double, and multiple modifications (comprising up to 4 amino acid substitutions) were included in the amino acid sequences of peptide 3973 tested. The presence of specific antibodies in the sera from 35 chronic chagasic patients living in Latin America was determined. The results show (Fig. 4) a drastic reduction in the recognition level when the 1st, 3rd, 8th, 9th, and 10th positions were mutated in the repeat, as the sensitivities dropped to less than 50% (6175 peptide), 20% (3964 peptide), 10% (6383, 3974, 6174, and 6382 peptides), and 5% (6172, 6381, 6385, and 6384 peptides). However, modification of amino acids located at positions

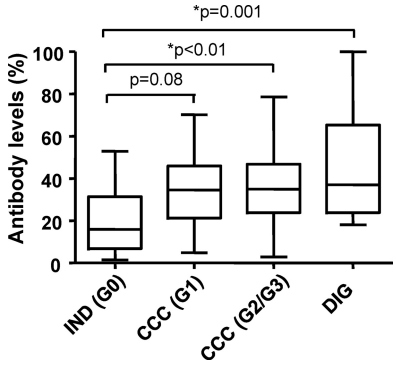


**FIG 3** Correlation analysis by Jonckheere-Terpstra test. The level of IgG-specific antibodies against the 3973 peptide in sera from 28 patients with the indeterminate form, 12 patients with the early cardiac form (G1), 26 patients with advanced cardiac form (G2-G3), and 21 patients with the digestive form were measured by ELISA. The antibody levels were correlated with clinical forms by the Jonckheere-Terpstra test. Statistically significant differences ($P < 0.05$) are indicated and marked with an asterisk. The lower and upper limits of the box indicate the 25th and 75th percentiles, respectively, the line within the box depicts the median, and the whiskers indicate the lower and upper adjacent values.

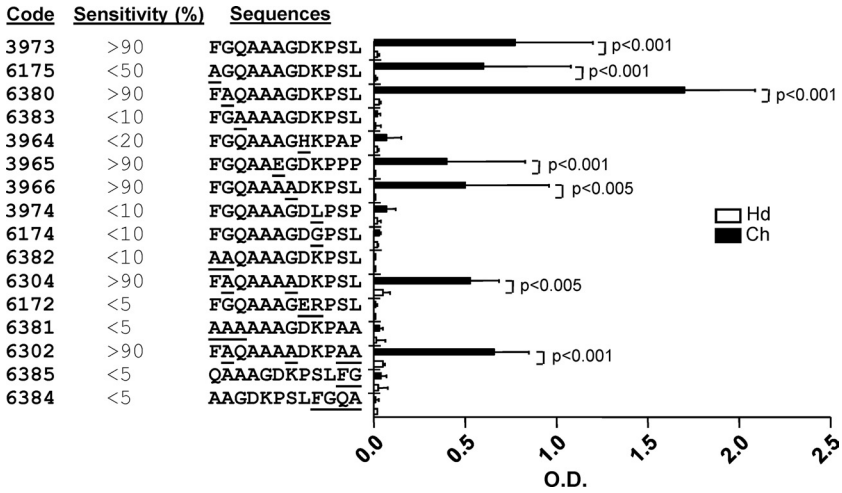


**FIG 4** Identification of minimal residues that conform to the antigenic epitope recognized by the chagasic patients' serum samples. Reactivity of sera from Chagas' disease patients against 15 12-mer-long peptides derived from the 3973 peptide contained in the protein TcCA-2. Mutated positions are underlined. The horizontal bars represent the median values with interquartile range of 35 serum samples from chagasic patients (■) and 20 serum samples from healthy donors (□). Data are expressed in optical density (O.D.) values. Statistically significant differences ($P < 0.05$) are indicated.

2 (peptide 6380), 6 (peptide 3965), and 7 (peptide 3966), including double mutations at positions 2 and 7 (peptide 6304), did not affect the level of recognition, as they were recognized by at least 90% of the ChD patients' sera. Peptide 6380 revealed the highest (optical density) reactivity of all the different peptides assayed. All these peptides share the amino acids phenylalanine (F) at the 1st position, glutamine (Q) at the 3rd position, aspartic acid (D) at the 8th position, lysine (K) at the 9th position, and proline (P) at the 10th position. The relevance of these amino acids at these particular positions was confirmed by synthesizing a peptide that maintains invariable the amino acids at positions 1, 8, 9, and 10 in peptide 3973 and bears alanine at the other positions. The pattern of recognition of peptide 6302 was similar to that of 3973 as an indication that the consensus sequence FXQXXXXDKPXX defines the minimal sequence structure required for a proper recognition. More than 90% of the sera recognized peptide 6302.

In order to determine whether there is any correlation between the reactivity detected against peptides 3973 and 6302, a paired correlation analysis was carried out. The results shown in Table 2 indicate the existence of a statistically significant correlation in the recognition of these peptides, with values of $r$ of 0.691 ($P < 0.001$) for chagasic patients, 0.568 ($P < 0.005$) for patients with cardiac clinical manifestations, and 0.578 ($P < 0.001$) for patients with digestive clinical manifestations.

**TABLE 2** Correlation between the reactivity of sera against peptides 3973 and 6302

| Subject group[a] | Correlation for 3973 vs 6302 | |
|---|---|---|
| | *P* value | *r* |
| Chagasic patients | <0.001 | 0.691 |
| IND | <0.001 | 0.556 |
| CCC | <0.005 | 0.568 |
| DIG | <0.001 | 0.578 |

[a] IND, chronic Chagas' disease patients with indeterminate clinical form of the disease; CCC, chronic cardiac Chagas' disease patients; DIG, chronic Chagas' disease patients with abnormalities in the gastrointestinal tract.

## DISCUSSION

Several serological methods for diagnosis of ChD have been developed and are being used across the world, as they have proven high rates of sensitivity ranging from 88 to 100% and specificity near 95% (26). The cross-reactivity of the mentioned methods is mainly due to the recognition of shared antigens present in *Leishmania* and *Trypanosoma rangeli* parasites (5). Given that a few infected patients do not seem to be properly diagnosed, WHO recommends that the diagnosis be made using two serological tests and positive results by the two tests be considered evidence of infection (11, 31). Many antigens of *Trypanosoma cruzi* that contain highly repetitive immunogenic amino acid motifs have been used in diagnostic tests for Chagas' disease. These repetitive motifs provide markedly improved specificity compared to that obtained using conventional tests (14). In the study described in the present paper, we analyzed the reactivity of sera from Chagas' disease patients at different clinical phases of the disease against five 12-mer peptides that correspond to different tandem repeats from the TcCA-2 immunodominant antigen of *T. cruzi* (4). The data presented show that three out of the five different repeats contained in a TcCA-2 membrane surface protein, peptide 3972 (sequence, FGQAAAGDKPPP), peptide 6303 (sequence, FGQAAAGDKP AP), and peptide 3973 (sequence, FGQAAAGDKPSL), are recognized with high sensitivity (>90%) by sera from symptomatic and nonsymptomatic chronic ChD patients. These peptides are not recognized by sera from patients in the orally acquired acute phase of the disease.

The level of recognition detected against each one of the assayed repeats is not related to the abundance of the epitopes in the TcCA-2 protein (GenBank accession number EAN97076), since the highest level of reactivity is directed against the 3973 epitope, which is the least represented repeat in the protein (2 repeats). A similar recognition level was observed with sera from different regions where ChD is endemic (laboratory data). It has been described that the homologous tandemly repetitive sequences present in the B13 antigen from *T. cruzi* (Y strain) can be presented for T-cell recognition in the context of at least three distinct HLA class

II molecules (1). Furthermore, our results also show that a single amino acid divergence in a particular position of the repeat (P in position 10 replaced by L or D in position 8 replaced by G) resulted in a dramatic reduction of the percentage of patients' sera that recognized the peptide, an indication of the essential role that certain amino acids may play in the conformation of the immunodominant antigenic epitope. Moreover, a dramatic reduction in the recognition level was also detected when the amino acids F, Q, and K at the first, third, and ninth positions were mutated in peptide 3973. However, amino acid substitution at positions 2 (G replaced by A), 6 (A replaced by E), 7 (G replaced by A), 11 (S replaced by P or A), and 12 (L replaced by P or A) are irrelevant in terms of the recognition level.

The 3973 epitope does not cross-react with sera from patients with leishmaniasis and other related infectious diseases, showing a degree of specificity higher than 98% when tested against the sera from ChD patients. Moreover, the level of recognition of the 3973 peptide by sera from patients at the symptomatic chronic phase involving cardiac or digestive alterations is higher ($P < 0.01$ and $P < 0.005$, respectively) than that by sera from patients at the indeterminate phase of the disease. The data presented here also show that the 3973 epitope is not recognized by the sera from individuals with SLE, rheumatoid arthritis, or celiac disease or patients with a nonchagasic cardiomyopathy. The results suggest the presence of the parasite in tissues from chronic ChD patients and its essential role in the pathogenesis of the chronic phase of this disease (20, 28, 33).

The higher level of 3973 epitope-specific antibodies observed in sera from symptomatic chronic ChD patients than in sera from asymptomatic patients may be associated with parasite proliferation in the infected tissues due to a breaking off of the fragile equilibrium between the host immune response and the proliferation of parasites in patients at the indeterminate phase of the disease. In fact, an association between the parasite load and sickness severity has been described (22). However, the possibility cannot be excluded that during the infection the mimicry of parasite antigens with host self-antigens might lead to immune confusion and activation of the response against particular antigenic epitopes of the parasite. Supporting this assumption, it has been described that $CD4^+$ T-cell clones that infiltrated the hearts of CCC patients cross-recognize human cardiac myosin and the *T. cruzi* B13 protein (17).

We believe that regardless of the cause of the variation in reactivity against the 3973 peptide during instauration and progression of chronic ChD, the results described here have an important value for the follow-up of patients with this disease and for the evaluation of tissue damage progression. In fact, a positive trend (Jonckheere-Terpstra test) was found between the level of reactivity against the 3973 peptide in sera from chronic ChD patients and the severity of the cardiac damage determined by the Kuschnir classification (G0 to G2/G3), as well as between patients with digestive alterations of ChD and patients at the indeterminate phase. Thus, the diagnostic technique described has demonstrated high sensitivity and specificity, and its results can correlate with the degree of pathology of chronic ChD and define when a ChD patient in the indeterminate phase may progress toward a phase with cardiopathy or digestive disorders. Currently, the treatment with benznidazole or nifurtimox has been shown to be effective in patients at the acute phase of the disease, but its efficacy in adult patients at the chronic phase is under consideration (21, 39). There is a pressing need for useful tools that may discriminate between different grades of disease severity to help the clinician to decide whether or not to treat a particular chronic ChD patient and to make a favorable posttreatment follow-up.

## ACKNOWLEDGMENTS

We thank M. E. Patarroyo (Fundación Instituto de Inmunología, Bogotá, Colombia) and Henry Bermudez, Maria Angelita Lorenzo, and Adriana Gauna (Instituto de Medicina Tropical, Venezuela) for providing some of the synthetic peptides, as well as L. Murcia and A. Iborra from the Hospital Virgen de la Arrixaca (Murcia, Spain) for their assistance in collecting biological samples. We also thank C. Juárez and M. A. Martínez from the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) for their help in collecting serum samples from patients suffering autoimmune diseases and A. López-Barajas (IPBLN-CSIC) for technical assistance in serological tests. M.C.T., O.N., B.A.d.N., and M.C.L. are members of the NHEPACHA net work (Latin America Network for Chagas' disease).

This study was supported by grants P08-CVI-04037PAI (Junta de Andalucía), BFU2010-1670 from Plan Nacional I+D+i (MICINN), RD06/0021/0014 and RD06/0021/1007-ISCIII-RETIC (MICINN, Spain), and FEDER. M.S. and B.C. were supported by grant FIS, 2009SGR385, from ISCIII (MICINN, Spain).

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